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Gene expression, cellular localisation and function of glutamine synthetase isozymes in wheat (*Triticum aestivum* L.)

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Abstract We present the first cloning and study of glutamine synthetase (GS) genes in wheat (*Triticum aestivum* L.). Based on sequence analysis, phylogenetic studies and mapping data, ten GS sequences were classified into four sub-families: *GS2* (*a*, *b* and *c*), *GS1* (*a*, *b* and *c*), *GSr* (*1* and *2*) and *GSe* (*1* and *2*). Phylogenetic analysis showed that the wheat GS sub-families together with the GS genes from other monocotyledonous species form four distinct clades. Immunolocalisation studies in leaves, stems and rachis in plants at flowering showed GS protein to be present in parenchyma, phloem companion and perifascicular sheath cells. In situ localisation confirmed that *GS1* transcripts were present in the perifascicular sheath cells whilst those for *GSr* were confined to the vascular cells. Studies of the expression and protein profiles showed that all GS sub-families were differentially expressed in the leaves, peduncle, glumes and roots. Expression of GS genes in leaves was developmentally regulated, with both GS2 and GS1 assimilating or recycling ammonia in leaves during the period of grain development and filling. During leaf senescence the cytosolic isozymes, GS1 and GSr, were the predominant forms, suggesting major roles in assimilating ammonia during the critical phases of remobilisation of nitrogen to the grain. A preliminary analysis of three different wheat genotypes showed that the ratio of leaf GS2 protein to GS1 protein was variable. Use of this genetic variation should inform future efforts to modulate this enzyme for pre-breeding efforts to improve nitrogen use in wheat.

Keywords Glutamine synthetase · Wheat · Senescence · Nitrogen · Gene expression · In situ hybridisation · Immunolocalisation

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Abbreviations

GS	Glutamine synthetase
QTL	Quantitative trait loci
DH	Doubled haploid
qRT-PCR	Real-time reverse transcription polymerase chain reaction
RACE	Rapid amplification of cDNA ends

Introduction

Nitrogen is assimilated into glutamine in plants through the action of the enzyme glutamine synthetase (GS; EC 6.3.1.2) (Hirel and Lea 2001). Several sources of ammonia exist in plant cells and its production has been shown to vary throughout plant development. To enable its central role of cellular ammonia assimilation, GS is controlled by a sophisticated regulatory system involving developmental, metabolic, and subcellular/cellular/organ specific cues (Miflin and Habash 2002). Understanding the physiological functions and regulations of this enzyme is central to our efforts to modulate nitrogen metabolism and to screen for germplasm with enhanced efficiency of nitrogen utilisation. This has become increasingly relevant in view of the growing environmental concerns arising from high fertiliser input agriculture.

GS exists in multiple enzyme forms with the chloroplastic isozyme encoded by one gene and the cytosolic encoded by 3–5 genes depending on the species. Studies have shown that both GS isozymes are regulated in a developmental manner in leaves (Tobin et al. 1985; Kamachi et al. 1991; Finnemann and Schjoerring 2000; Habash et al. 2001) and have different metabolic roles. During the vegetative stage, GS2 is the predominant isozyme in the leaf mesophyll cells, where it assimilates ammonia originating from nitrate reduction and photorespiration (Wallsgrove et al. 1987; Tobin and Yamaya 2001). Cytosolic GS has multiple metabolic functions such as assimilating ammonia into glutamine for transport and distribution throughout the plant and immunolocalisation studies in tobacco (Brugière et al. 1999), pine (Canovas et al. 2007), potato (Pereira et al. 1995) and in rice (Sakurai et al. 1996; Tabuchi et al. 2005) have shown predominant vascular location in different organs. During leaf senescence cytosolic GS fulfils a key function in the assimilation and recycling of ammonia generated from various catabolic processes (Masclaux et al. 2001). This role is particularly important after anthesis and during grain development and filling in cereals when nitrogen is remobilised to the reproductive sinks. To date, however, only two studies have firmly assigned cytosolic GS genes, *Os-Gln1;1* in rice (Tabuchi et al. 2005) and *Gln1.3*, *Gln1.4* in maize (Martin et al. 2006) to leaf related remobilisation of assimilate to the grain.

To achieve these multiple non-overlapping roles, GS isozymes need to be regulated at many levels including transcription, mRNA stability, polypeptide synthesis and targeting to the subcellular localisation, assembly of subunit into an active enzyme, post-translational modification of the enzyme and protein turnover (Forde and Cullimore 1989; Kamachi et al. 1991, 1992; Li et al. 1993; Ortega et al. 1999; Finneman and Schjoerring 2000; Tobin and Yamaya 2001; Ishiyama et al. 2004b). Whilst studies on GS regulation in several species have shown some common regulatory mechanisms, some also highlighted differences particularly in gene expression, protein and enzyme activity levels (McNally et al. 1983; and latter references). Variation in GS regulation may be due to the occurrence of either C_3 or C_4 metabolism in leaves or inherently different nitrogen economies due to environmental adaptation. For example, maize continues to have substantial nitrogen uptake throughout the reproductive period whilst this is diminished for species such as rice and wheat and this may have a direct bearing on the dynamics of carbon and nitrogen remobilisation during grain filling (Limami and De Vienne 2001; Hirel et al. 2005, 2007). Therefore, it is important to study the role of each GS gene in a variety of plant species and particularly so for crops.

Recently, differences in GS protein amount and/or activity have been established in crop genotypes and such data can be exploited through QTL analysis to improve our understanding of the contribution of GS to complex agronomic traits relating to nitrogen use and yield. A significant correlation has been observed between grain number/size and a locus for cytosolic GS protein content in rice (*Os-Gln1;1*; Yamaya et al. 2002; Obara et al. 2004). This was recently confirmed by work on rice mutants (Tabuchi et al. 2005). In maize, coincidences in QTLs were found for GS activity, cytosolic GS locus (*Gln1)* and grain yield (Hirel et al. 2001, 2007; Galais and Hirel 2004). Furthermore, in wheat, GS activity (GS2 and GS1) was shown to be positively correlated to grain and stem nitrogen content (Habash et al. 2007). These studies highlight the importance of the cytosolic GS genes in determining several aspects of nitrogen use traits in cereal crops with potential implications for pre-breeding and agriculture. We present in this paper the cloning, characterization and cellular localisation of the GS gene family in hexaploid wheat with a major focus on developmentally specific studies. Results are discussed in the context of our current knowledge of GS gene expression and function in the two other major cereals, rice and maize.

Materials and methods

Plant material and growth conditions

Wheat plants cv. Cadenza (a former UK commercial variety) and doubled haploid (DH) lines 90 and 91 (derived

from the cross Chinese Spring (CS) × SQ1; Quarrie et al. 2005) were grown in soil in 2 L pots at one plant per pot in a fully randomised design in glasshouses. Soil was supplemented with 2 kg Osmocote plus m^{-3} (Scotts Company, UK) and 0.5 kg PG mix m^{-3} (Hydro Agri, UK). N was supplied at 7% in the osmocote mix and 8.5% in the PG mix while nitrate was added at 8 and 5.5%, respectively, to give 1.5 gN per plant. DH lines were vernalised for 4 weeks at 4°C prior to transplanting to glasshouses and grown under 350 µmol m^{-2} s^{-1} photon flux density and a 18/15°C (16/8 h) day/night temperature regime.

Heterologous complementation and cloning of wheat GS genes

A wheat root cDNA library (Schachtman and Schroeder 1994) was used for complementation of a haploid yeast (*Saccharomyces cerevisiae*) strain Δgln1 (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, YPR035w::kanMX4) auxotroph for glutamine that had been generated by sporulation from the diploid yeast Y25454 (http://www.uni-frankfurt.de/fb15/mikro/euroscarf) by standard procedures. The yeast was grown on either complex medium (YPD) supplemented with 0.1% w/v glutamine or on synthetic medium composed of 2% carbon (SD, glucose or SG, galactose), yeast nitrogen base without amino acids and ammonium (Difco), 50 mM succinic acid pH 5.5 and 0.1% w/v glutamine as nitrogen source supplemented with histidine, leucine, methionine and uracil. For heterologous complementation of glutamine auxotrophy and cloning of wheat GS genes, aliquots of yeast transformed with the cDNA library were plated on synthetic medium containing galactose (SG), and ammonium as nitrogen source, while omitting glutamine. Complementing clones were identified by plasmid sequencing.

Cloning of wheat GS isoforms by 5′ and 3′ rapid amplification of cDNA ends (RACE)

Further sequences, GS1 (a, b and c) and GS2 (a, b and c) were obtained by PCR cloning. Specific primers (Table S1) were designed according to the TIGR database sequences. First, PCR was carried out using the cDNA library as template with gene specific primers located in the 3′ UTR and a plasmid specific primer (T7) as forward (5′ RACE). PCR conditions were as follow: 94°C initial denaturation for 3 min, 30 cycles of 94°C 1 min, annealing temperature specific for each primer pair (Table S1) for 1 min and 72°C 3 min, with a final elongation at 72°C for 10 min. After sequencing of the resulting products, a second round of PCR using nested primers was performed. To clone the 3′ UTR region (3′RACE), gene specific primers were chosen in the 5′ UTR and an oligo $d(T)_{18}$-N was used as reverse primer (Table S1).

Leaf tissue sections

For morphological and structural observations, leaf sections were taken from tillers supporting grain at milk (Zadoks 73) and early dough (Zadoks 83) (Zadoks et al. 1974). Sections, 1.2 mm wide across the width of the leaf, were cut from the centre of the organ, fixed and embedded in Lowikryl K4M as described elsewhere (Lopes et al. 2006). Transverse-sections, 1 µm thick, were cut with a microtome Leika MFS (Vienna), stained with methylene blue and photographed with a light microscope (Olympus BHZ-UMA, Japan), coupled with a digital camera (JVC TK 1270, Germany).

Immunohistochemistry

The middle parts of the flag leaf, stem, glumes and rachis from plants at flowering (Zadoks 60) were fixed in freshly prepared 1.5% w/v paraformaldehyde dissolved in 0.1 M phosphate buffer pH 7.4 containing 0.05% v/v tween20, for 4 h at 4°C. The material was dehydrated in an ethanol series (final concentration 90% ethanol) then embedded in LR White resin (Polysciences). Polymerisation was carried out in gelatine capsules at 54°C. Ultra thin sections (70 nm) were probed with a polyclonal antibody raised against chloroplastic GS (Hirel et al. 1984) and treated as described by Kichey et al. (2005) and observed with a CM12 electron microscope (Philips, Eindoven, The Netherlands) at 100 kV.

In situ hybridisation

Leaf, stem, rachis and glumes from plants at flowering (Zadoks 60) were collected and immediately frozen in liquid nitrogen. The middle part of each organ was cut with a cryostat (Leica CM1900) at −15°C. Sections (10 µm) were collected on glass slides, fixed in freshly prepared 4% w/v paraformaldehyde dissolved in 0.1 M sodium phosphate buffer pH 7.4 for 30 min at room temperature and followed by a wash in sodium phosphate buffer. Tissues were then treated in 1% v/v acetic anhydride in 0.1 M triethanolamine pH 8 for 10 min, washed in sodium phosphate buffer and finally dehydrated through an ethanol series (30, 50, 85, 95, and 100%). Incubation of dehydrated sections with digoxigenin-labeled RNA probes and revelation of label were performed as described by Martin et al. (2006), except that sections were incubated with 30 ng for 12 h at 55°C.

Fragments of the 3′ non-coding coding region of *GSr1* (199 bp) and *GS1* (187 bp) were amplified by PCR using

the corresponding cDNA clones and cloned into the *SmaI* site of the pAMP1 vector (Gibco-BRL, Gaithersburg MD, USA), which was used to make digoxigenin-labeled riboprobes for in situ hybridization. The oligonucleotides used to amplify the 3′ non-coding region specific to each of the three GS genes were for *GSr1*: left-GATCATG ACACCCAGGCTCT; right-CGGAATGCGAAAACAAC ATT and for *GS1*: left-CGATGATCAGTGATGGGTTG; right-CCACCCAGACGAGAGAAG. Digoxygenin-labeled riboprobes were generated using DIG/RNA labeling SP6/T7 labeling (Boehringer, Mannheim, Germany). For the analysis of GS genes expression, both sense and antisense probes were used in parallel hybridizations with comparable tissue sections.

Tissue extraction, glutamine synthetase assays and Western blot analysis

Plant tissue was ground under liquid nitrogen to a fine powder and 0.1 g material was extracted in 1 ml of extraction buffer (pH 7.6) containing 100 mM triethanolamine, 1 mM EDTA, 10 mM $MgSO_4$, 5 mM glutamate, 10% v/v ethylene glycol, 10 µM leupeptin at pH 7.6 and 6 mM DTT. The crude extract was centrifuged at 21,000*g* for 30 min at 4°C. GS activity was measured using the synthetase assay based on the method described by Lea et al. (1990) and optimized for wheat leaves as follows. 100 µl of crude leaf extract was added to 380 µl of assay mix which consisted of 100 mM TEA, 80 mM glutamate, 6 mM hydroxylamine HCl, 20 mM $MgSO_4$, 4 mM EDTA at pH 7.6. The reaction was started by the addition of 20 µl of 0.2 M ATP at pH 7.6. After 10 min of incubation at 30°C, the reaction was stopped by the addition of 500 µl of ferric chloride reagent (0.24 M TCA, 0.1 M ferric chloride, 1.0 M HCl). Samples were then centrifuged at 10,000 *g* for 5 min and absorbance read at 505 nm. Controls consisted of all components except ATP. The amount of product formed was quantified against an authentic glutamylhydroxamate (Sigma) standard curve. Polypeptides from the crude extract were separated on 10% acrylamide gels and Western blot analyses were performed as described in Habash et al. (2001) using a GS specific antibody raised against root nodule GS1 at Rothamsted (Cullimore and Miflin 1984) and a Rubisco antibody raised against wheat rubisco also at Rothamsted (Khan et al. 1999). Broad range pre-stained standards (Bio-Rad) were used as markers.

Chlorophyll and proteins measurements

Chlorophyll was determined in leaf extracts prepared in 80% acetone according to Vernon (1960). Soluble protein content was measured in leaf extracts according to Bradford (1976).

RNA extraction and cDNA synthesis

Total RNA was isolated from wheat tissues using the TRIZOL Reagent (Invitrogen) according to manufacturer's instruction. Prior to cDNA synthesis (RETROscript kit, Ambion), genomic DNA contamination was removed by a TURBO DNAse treatment (Ambion).

Measuring GS mRNA abundance by real-time RT-PCR

Real-time RT-PCR (qRT-PCR) assays were performed with the 7500 Real-Time PCR system (Applied Biosystem) using Platinum® Quantitative PCR SuperMix-UDG (Invitrogen). The relative abundance of GS gene transcripts (target genes) from the different samples was calculated using the mathematical model proposed by Pfaffl (2001), which included a normalisation to the abundance of 18S rRNA (endogenous control gene) and to a calibrator sample. 18S rRNA was chosen as an endogenous control gene since it was shown to be suitable for studies on plant gene expression during senescence (Guo and Gan 2006) and in different plant tissue types (Jain et al. 2006). The algorithms also included correction in mRNA abundance to variation in gene amplification efficiency for the target and endogenous control gene. The gene amplification efficiency was estimated according to Ramakers et al. (2003). The calibrator sample is defined for each set of experiments in legends to figures and the amount of RNA was kept constant for each experiment, which enabled comparisons between experiments. Specific primers and dual-labelled fluorescence probes were designed using the Primer Express Software v2.0 (Applied Biosystems) and details are presented in Table S2.

Phylogenetic analysis

The amino acid sequences deduced from the cDNAs were aligned using the ClustalX software (Thompson et al. 1997). The clustal alignment was submitted to phylogenetic analysis using the interface to the PHYLogenetic Inference Package (version 3.5) software at www.hgmp.mrc.ac.uk/Registered/Webapp/PIE. Phylogenetic trees obtained with the maximum parsimony, neighbour-joining and maximum likelihood methods had an overall similar topology. Bootstrap analysis with 100 replicates was performed to assess the statistical reliability of each branch.

Statistical analysis

Analysis of Variance (ANOVA) was applied to the data using GENSTAT version 9.0 software. Natural log transformation of the data was used where necessary to account for heterogeneity of variance across the treatments. If the overall difference between treatments was significant ($P < 0.05$), the statistical significance of differences between treatments was assessed using the least significant difference (l.s.d) at the 5% level on the residual degrees of freedom from the ANOVA.

Results

GS gene sequences from hexaploid wheat

A total of ten GS gene sequences were cloned from wheat and the sequences of the full-length cDNA deposited in GenBank (Table 1). Comparison of nucleotide and amino acid sequences (Table 2) suggest that all GS sequences from wheat can be classified in four sub-families: *GS2* (*a*, *b* and *c*), *GS1* (*a*, *b* and *c*), *GSr* (*1* and *2*) and *GSe* (*1* and *2*). The sequences *GS2a*, *GS2b* and *GS2c* encoded the chloroplast-located isoforms as defined by the presence of the transit peptide, whereas *GS1a*, *GS1b*, *GS1c*, *GSr1*, *GSr2*, *GSe1* and *GSe2* lacked this specific targeting motif and hence encode cytosolic isoforms (Fig. 1). The length of the open reading frame and the polypeptide M_r and pI were similar within each GS sub-family (Table 1). The wheat GS amino acid sequences were very similar with percentage identity ranging from 71.8% to 99.7% (Table 2). Furthermore, sequence alignments highlighted that residues His-249, Asp-56, Glu-297, Cys-92, Cys-303 and Cys-368 were conserved in all wheat GS sequences (Fig. 1).

Three sub-families of cytosolic GS in monocotyledonous species

Phylogenetic analysis identified four monophyletic groups for GS polypeptide sequences in monocotyledonous species (Fig. 2). Sequences encoding chloroplastic GS formed clade A, which also contained GS2 sequences from dicots. Clades D, B and C were represented respectively by *GS1* (*a*, *b* and *c*), *GSr* (*1* and *2*) and *GSe* (*1* and *2*). Without exception, each cytosolic GS sequence from a monocot belonged to one of these three sub-families. However, the position of cytosolic GS from dicotyledonous species was less certain. For each wheat GS sequence we could identify at least one orthologous sequence belonging to the maize or rice GS family. Bread wheat is composed of three genomes and it is possible that the identified sequences correspond to homeoalleles within the wheat GS gene sub-families. The recent mapping of GS clones to specific chromosomes confirmed that *GS2*, *GS1*, *GSr*, and *GSe* group are located on chromosomes 2, 6, 4 and 4, respectively (Habash et al. 2007). Assigning specific homeoalleles for the isolated clones in this study, obtained from another genetic background, will require further experimental work.

Tissue and cellular localisation of GS

Immunolocalisation and in situ studies showed that GS proteins were present in specific cell types in plants at flowering. For the immunolocalisation experiments, a

Table 1 Details and accession numbers for wheat GS cDNAs available in GenBank

Name	Accession number	Length (bp)	ORF length (bp)	Number of AA[a]	M_r[b] (kDa)	pI[b]
GS2a	DQ124212	1,713	1,283	427	46.69	5.42
		Mature peptide[c]		380	41.76	4.96
GS2b	DQ124213	1,593	1,283	427	46.64	6.04
		Mature peptide[c]		380	41.71	5.14
GS2c	DQ124214	1,689	1,283	427	46.70	5.75
		Mature peptide[c]		380	41.77	5.02
GS1a	DQ124209	1,292	1,071	356	39.20	5.41
GS1b	DQ124210	1,496	1,071	356	39.21	5.41
GS1c	DQ124211	1,223	1,071	356	39.24	5.41
GSr1	AY491968	1,397	1,065	354	38.73	5.35
GSr2	AY491969	1,341	1,065	354	38.66	5.34
GSe1	AY491970	1,299	1,089	362	39.48	5.53
GSe2	AY491971	1,278	1,089	362	39.47	5.66

[a] Number of amino acids residues in the polypeptide

[b] M_r and pI values were estimated using the web based server at http://ca.expasy.org/tools/pi_tool.html

[c] The mature peptide was estimated with a transit peptide of 47 residues long

Table 2 Comparison between wheat GS amino acid and nucleotide sequences using the EMBOSS::needle global alignment tool (http://www.ebi.ac.uk/emboss/align)

		Amino acid identity									
		GS2a	*GS2b*	*GS2c*	*GSe1*	*GSe2*	*GSr1*	*GSr2*	*GS1a*	*GS1b*	*GS1c*
Nucleotide identity	*GS2a*		97	98	70	70	73	73	74	74	73
	GS2b	94		99	71	70	74	74	75	75	74
	GS2c	94	95		72	71	75	75	75	75	75
	GSe1	56	56	56		97	81	81	81	81	80
	GSe2	57	57	58	92		80	79	80	80	80
	GSr1	55	55	55	67	67		99	81	81	81
	GSr2	55	56	56	66	67	93		81	81	81
	GS1a	57	57	57	69	70	67	67		99	99
	GS1b	52	49	49	66	68	65	65	95		99
	GS1c	60	60	60	70	70	73	71	97	98	

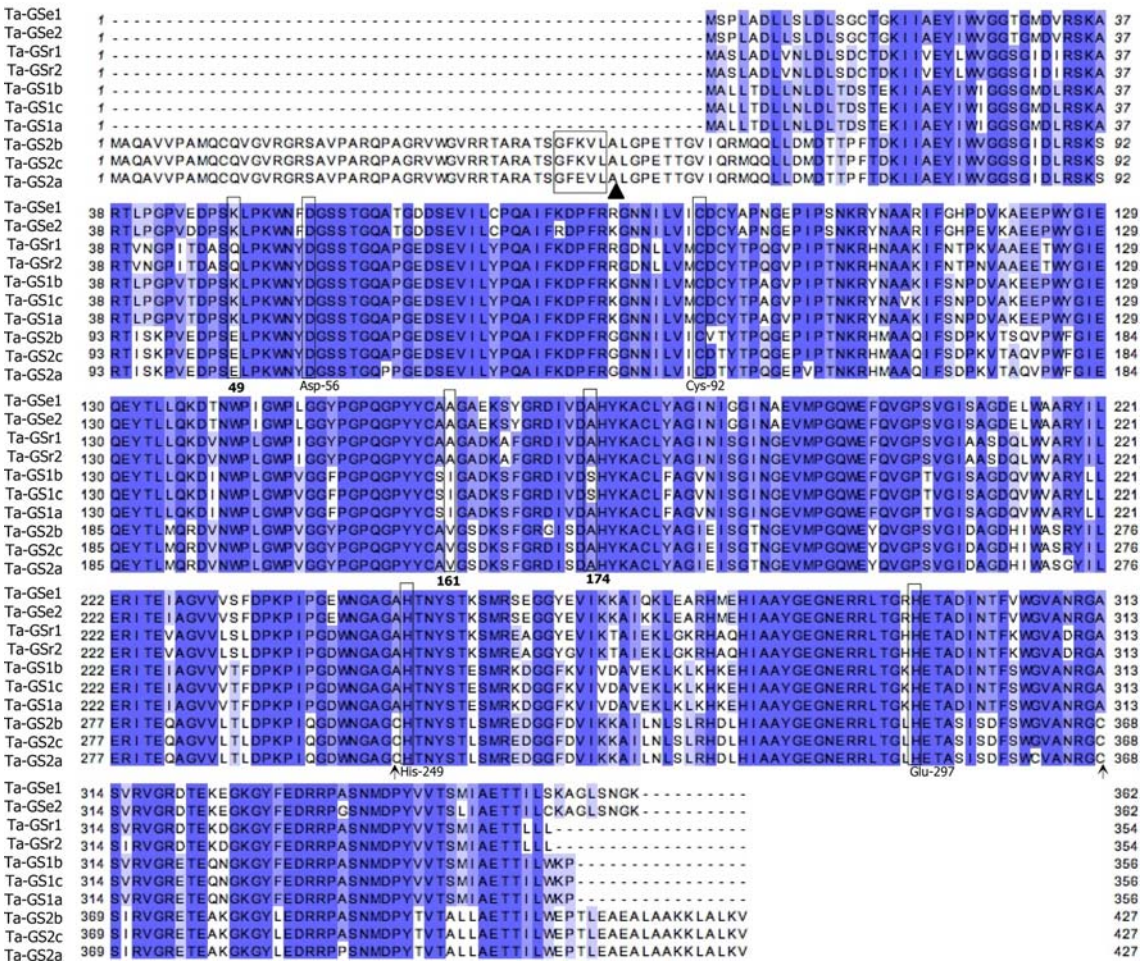

Fig. 1 ClustalX multiple alignment of wheat glutamine synthetase amino acid sequences. The conserved motif indicating the position of the cleavage site for the transit peptide are delimited by a box on GS2 sequences while the large arrow indicates the most likely processing site for the GS2 pre-sequence. The two small arrows indicate the two cysteine residues conserved only in GS2 (Choi et al. 1999). Residues conserved across all GS sequences and which are of particular interest are highlighted by a box. Residues 49, 161 and 174 are important in determining the substrate affinity of each isoform (Ishiyama et al. 2006)

polyclonal GS antibody was used which highlighted both GS1 and GS2 proteins (Hirel et al. 1984). In a mature flag leaf, immunolabelling with anti GS antibody showed the presence of label in the plastid of mesophyll parenchyma (Fig. 3a) and in the plastid of parenchyma cells in the perifascicular sheath surrounding the vascular bundles

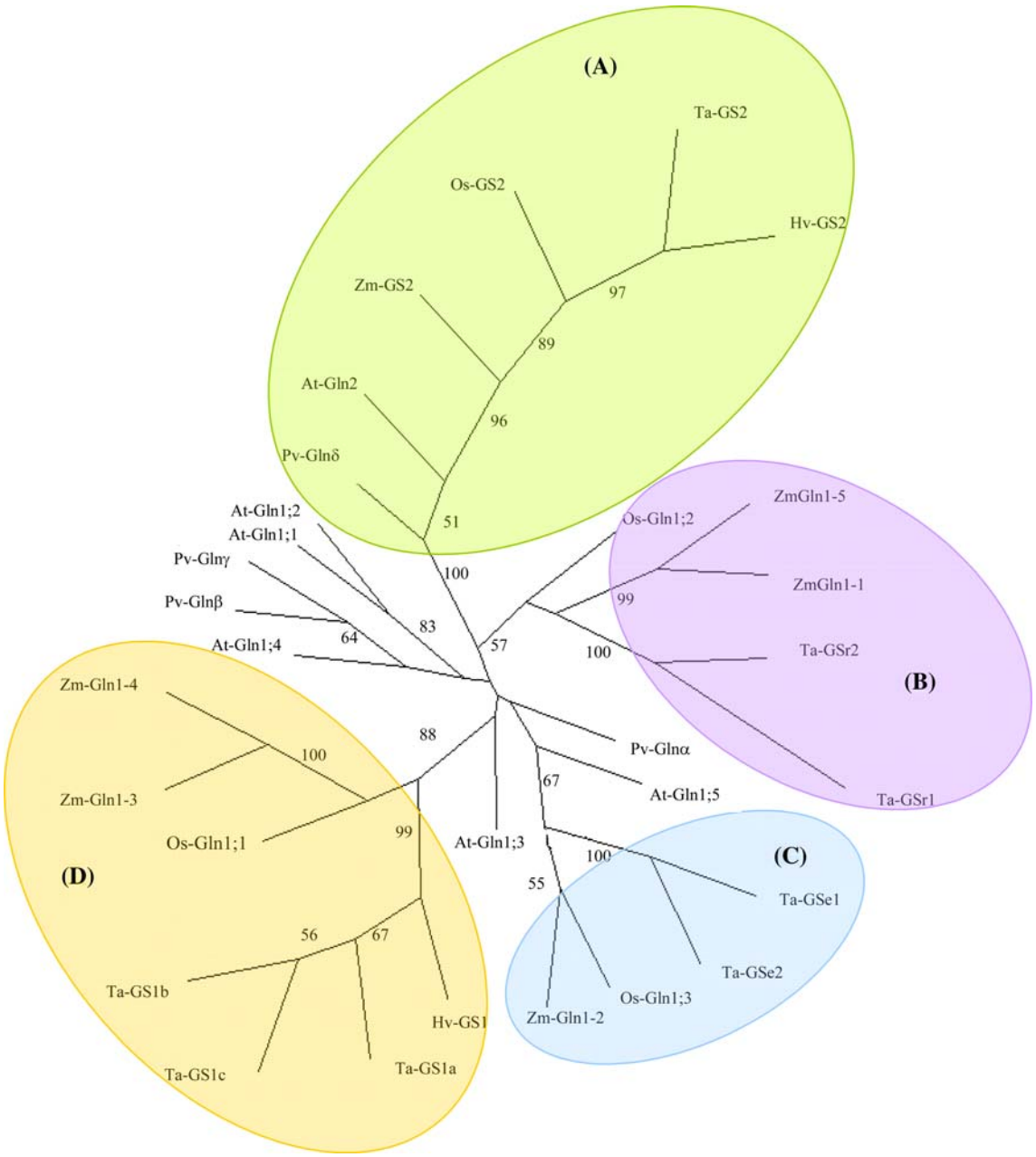

Fig. 2 Unrooted phylogenetic tree of GS protein sequences from plants. Analysis was carried out using the PIE interface (www.hgmp.mrc.ac.uk/Registered/Webapp/PIE) to the phylogeny inference package (PHYLIP) version 3.5. The proml analysis was used with a JTT model for amino acid change and the tree calculated by the maximum likelihood method. The reliabilities of each branch point were assessed by bootstrap analysis (100 replicates). Bootstrap values are displayed on the tree. Ta-GS2a, AAZ30060; Ta-GSe1, AAR84349; Ta-GSe2, AAR84350; Ta-GSr1, AAR84347; Ta-GSr2, AAR84348; Ta-GS1a, AAZ30057; Ta-GS1b, AAZ30058; Ta-GS1c, AAZ30059; Os-GS2, CAA32462; Os-Gln1;1, CAA32461; Os-Gln1;2, CAA32460; Os-Gln1;3, AAK18848; Zm-GS2, CAA46724; Zm-GS1-1, CAA46719; Zm-GS1-2, CAA46720; Zm-GS1-3, CAA46721; Zm-GS1-4, CAA46722; Zm-GS1-5, CAA46723; Hv-GS2, CAA37643; Hv-GS1, CAA48830; At-Gln1;1 (At5g37600), NP_198576; At-Gln1;2 (At1g66200), NP_176794; At-Gln1;3 (At3g17820), NP_188409; At-Gln1;4 (At5g16570), NP_568335; At-Gln1;5 (At1g48470), NP_175280; At-Gln2 (At5g35630), AAB20558; Pv-Gln-δ, CAA31234; Pv-Gln-α, CAA27632; Pv-Gln-β, CAA27631; Pv-Gln-γ, CAA32759

(Fig. 3c); this is GS2. Further label was also detected in the cytosol of leaf phloem companion cell (Fig. 3b) and in the cytosol of leaf parenchyma and mestome sheath cells surrounding the vascular bundles (Fig. 3c); this is cytosolic GS. In situ localisation, corresponding to the blue signal, showed that *GS1* label was present in the cytosol of parenchyma and the mestome sheath cells surrounding the vascular cells (Fig. 3d, e). There was also some label in the

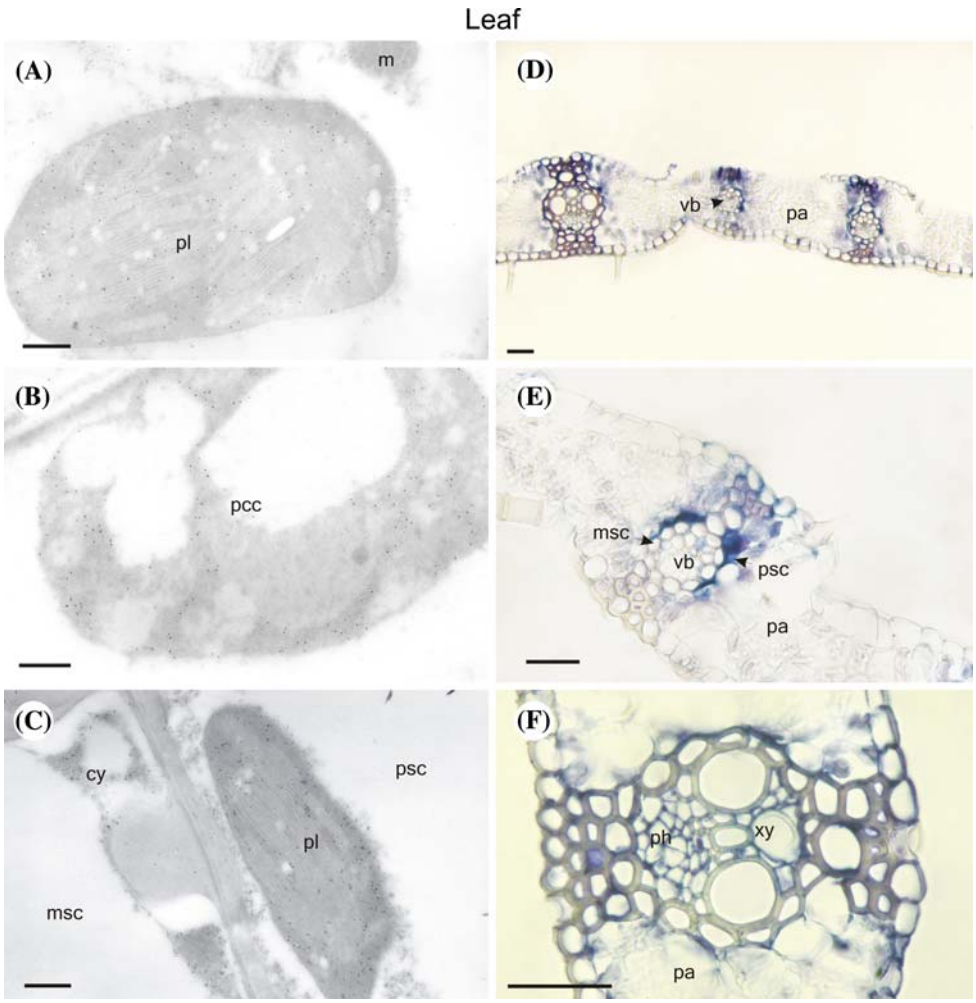

Fig. 3 Cellular and tissue localisation of GS in flag leaf sections at flowering (Zadoks 60). Transmission electron microscopic immunolocalisation of GS in mesophyll parenchyma (**a**) phloem companion cells (**b**) and perifascicular sheath cells (**c**). In situ localisation of anti GS1 probes in parenchyma surrounding vascular cells (**d**) and magnified (**e**); and of anti GSr probes in vascular cells (**f**). Cy, cytosol; m, mitochondria; msc, mestoma sheath cell; pa, parenchyma; pcc, phloem companion cell; ph, phloem; pl, plastid; psc, parenchyma sheath cell; vb, vascular bundle; xy, xylem. Bars: 0.5 µm (**a**, **b**, **c**) and 100 µm (**d**, **e**, **f**)

sclerenchyma cells (Fig. 3d, e) but results with a sense probe to *GS1* showed the presence of some label in those cells indicating lack of specificity in that particular region (Fig. S1). In situ localisation with anti *GSr* showed the label to be predominantly in the vascular phloem cells and the parenchyma cells close to the xylem (Fig. 3f).

In stem and rachis, the vascular bundles have a different spatial distribution when compared to the leaf with the presence of an outer and inner ring of vascular bundles embedded amongst predominantly cortical cells (Figs. 4d, 5d). Immunolabelling showed the presence of label in the parenchyma plastid (Figs. 4a, 5a), the cytosol of phloem companion cell (Figs. 4b, 5b) and the cytosol of the perifascicular sheath cells close to vascular cells (Figs. 4c, 5c). In stems and rachis, in situ localisation showed that *GS1* label was present in parenchyma cells near the vascular tissue on the peripheral side of the organ below the epidermis (Figs. 4e, 5e) whilst the probe against *GSr* was predominantly in vascular cells (Figs. 4f, 5f). Glume tissue was also examined and the results were similar to those of the stem and rachis (data not shown). Controls using pre-immune serum for immunolocalisation showed lack of label (Fig. S1a) and sense probes for in situ experiments showed some label in schlerenchyma cells in leaves but none in stem tissue (Fig. S1b, c).

Tissue specific expression of wheat GS genes

GS2 mRNA was detected in all wheat tissues examined, with the highest level in mature leaf and peduncle tissues (Zadoks 60), to a lesser extent in photosynthetic glumes (Zadoks 75) and with some expression also measured in the root (Zadoks 12) (Fig. 6a). *GS1* mRNA was also detected in all tissues, with transcripts being most abundant in glume (Zadoks 75) and flag leaf tissue (Zadoks 60) and least abundant in seedling leaves (Zadoks 12). *GSr* transcripts were prevalent in glume (Zadoks 75) and root (Zadoks 12) tissue but lower levels were measured in leaf tissue especially at a young developmental stage (Zadoks 12). The level of *GSe* mRNA was very low

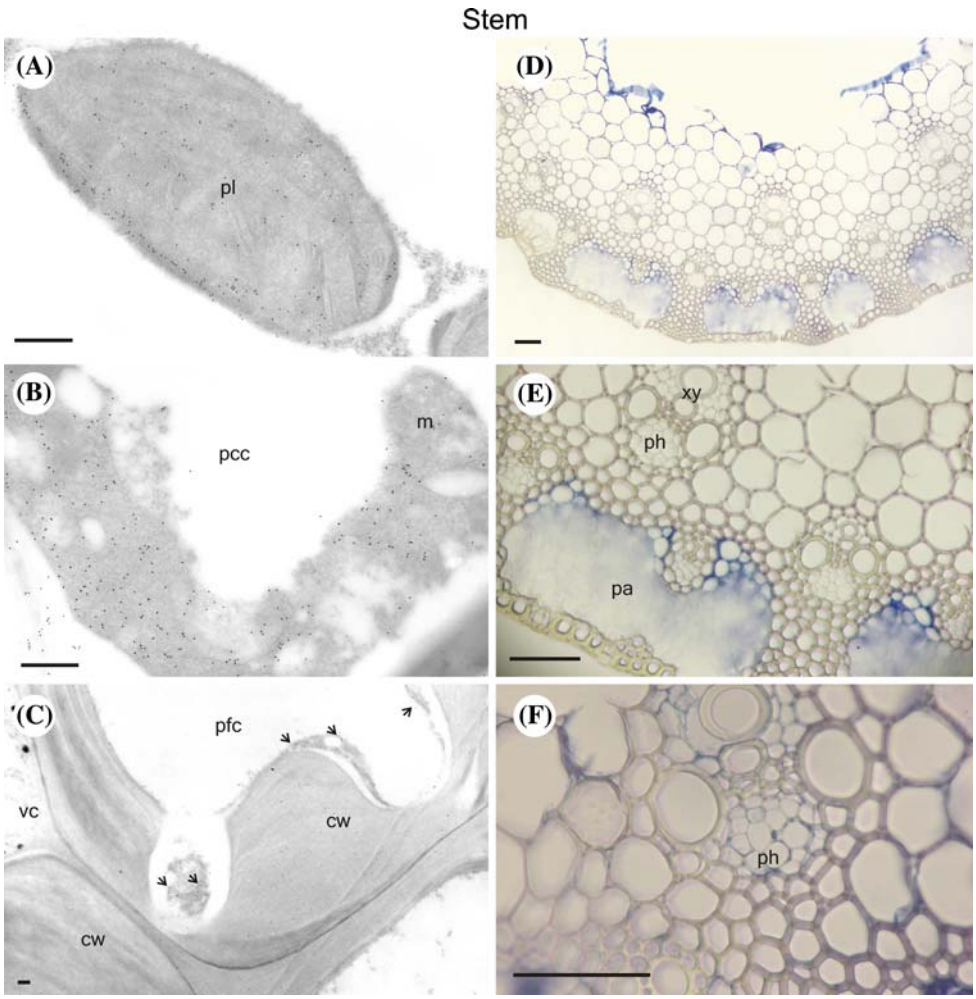

Fig. 4 Cellular and tissue localisation of GS in stem sections at flowering (Zadoks 60). Transmission electron microscopic immunolocalisation of GS in parenchyma (**a**) phloem companion cells (**b**) and perifascicular sheath cells (**c**). In situ localisation of anti GS1probes in parenchyma near peripheral vascular cells (**d**) and magnified (**e**); and of anti GSr probes in vascular cells (**f**). Cw, cell wall; m, mitochondria; pa, parenchyma; pcc, phloem companion cell; pfc, perifascicular cell; ph, phloem; pl, plastid; vc, vascular cell; xy, xylem. Bars: 0.5 μm (**a**, **b**, **c**) and 50 μm (**d**, **e**, **f**)

compared to that of other GS genes and showed no prevalence in any tissue examined. The same plant tissue was probed for GS polypeptide content using an antibody which recognized chloroplastic and cytosolic GS isoforms; at ca. 43 kDa and ca. 39 kDa, respectively (Fig. 6b). The bands of higher and lower molecular weight correspond to chloroplastic GS and cytosolic GS, respectively (Forde and Cullimore 1989). The results showed that GS2 polypeptides were prevalent in photosynthetic tissue with the highest amount detected in flag leaves at anthesis (Zadoks 60). GS2 polypeptide content mirrored the changes in mRNA abundance for most tissues except for the root which showed no polypeptide on blots. In root (Zadoks 12) and peduncle (Zadoks 60), cytosolic GS (ca. 39 kDa) was the prevalent isozyme with equivalent amounts reported in glume. Blots in Fig. 6b could not distinguish between the three cytosolic GS gene products, but it seemed that the polypeptide profile of cytosolic GS followed most closely that of *GSr* mRNA especially for root and peduncle. Total GS activity per unit of soluble protein was comparable for all tissue types (data not shown).

GS sub-families vary during leaf development and senescence

Flag leaf total RNA, chlorophyll, soluble protein and total GS activity expressed on a fresh weight basis showed very little change from booting stage (Zadoks 40) to anthesis (Zadoks 60) (Fig. 7a–d). After anthesis, these parameters gradually declined showing statistically significant changes in flag leaves supporting grains from early milk stage (Zadoks 73) onwards. The changes in leaf total GS activity expressed on a fresh weight basis (Fig. 7d) correlated well with the decline in leaf soluble protein (Fig. 7b). The large subunit of Rubisco (RbcL) followed the same pattern of change and showed a loss in leaves supporting grain from early milk (Zadoks 73) onwards whilst the small subunit of Rubisco (RbcS) only showed a large drop at the final developmental stage (Fig. 7e). The expression of GS genes at the mRNA level was studied in the same leaf tissue. Results showed that *GS2* transcript remained unchanged in relation to 18S rRNA in leaves from tillers at booting stage through to grain at early milk (Zadoks 73) but showed a

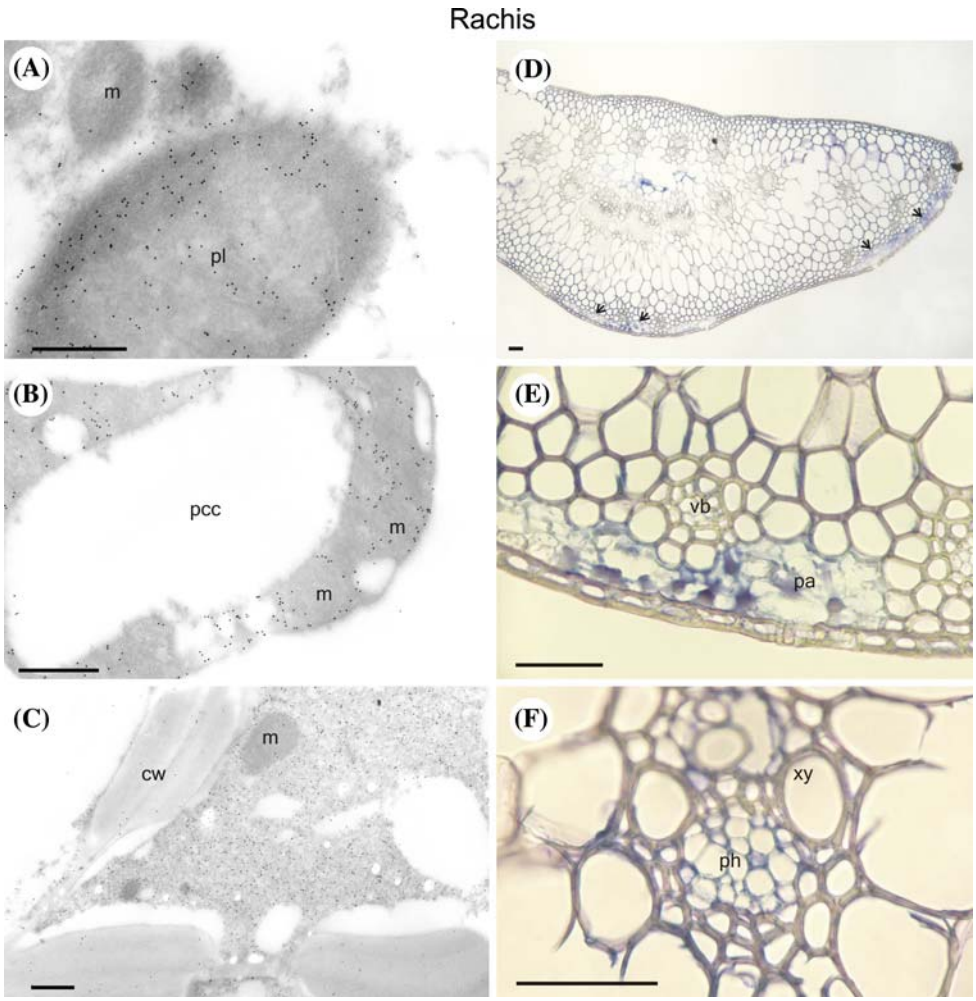

Fig. 5 Cellular and tissue localisation of GS in rachis sections at flowering (Zadoks 60). Transmission electron microscopic immunolocalisation of GS in parenchyma (**a**) phloem companion cells (**b**) and perifascicular sheath cells (**c**). In situ localisation of anti GS1 probes in parenchyma cells (**d**) and magnified (**e**); and of GSr in vascular cells (**f**). Cw, cell wall; m, mitochondria; pa, parenchyma; pcc, phloem companion cell; ph, phloem; pl, plastid; vb, vascular bundle; xy, xylem. Bars: 0.5 μm (**a**, **b**, **c**) and 100 μm (**d**, **e**, **f**)

downward trend in leaves supporting grain at medium milk (Zadoks 75) and soft dough (Zadoks 85) (Fig. 7f). The amount of *GS1* transcripts showed an upward trend in relation to 18S rRNA in leaves supporting ears from booting stage to medium milk; this was only statistically significant between leaves at Zadoks 40 and at Zadoks 75. Further, we also measured a decline in *GS1* mRNA abundance in leaves at the soft dough stage (Zadoks 85) but this was not statistically significant in our experiment (Fig. 7f). The level of *GSr* mRNA was maintained in relation to that of 18S rRNA in leaves supporting tillers from booting stage to medium milk but was doubled at the final stage of grain soft dough (Zadok 75; Fig. 7f). *GSe* mRNA levels were very low in comparison to other GS genes and showed no distinctive pattern (Fig. 7f). Western blots analysis on the same leaf extracts, normalised to soluble protein, showed GS2 to be the predominant isoform in leaves supporting ears at the booting, ear emergence and anthesis stages after which it declined gradually and became very low at the later stage of grain filling (Fig. 7g). This polypeptide profile followed the same trend as was seen for *GS2* mRNA levels (Fig. 7f). Results also showed that the amount of cytosolic GS polypeptides (which may comprise the product of all three cytosolic genes *GS1*, *GSr* and *GSe*) gradually increased in leaves supporting the developing grain (Fig. 7g). The profile of RbcL protein (Fig. 7h) showed a gradual decline in leaves supporting ears during the filling period and was thus similar to the profile of changes in GS2 protein (Fig. 7g). Total GS activity expressed on a soluble protein basis was maintained in wheat leaves throughout all these stages of grain fill but declined by 30% at the soft dough stage (Fig. 7i).

Leaf cellular organisation during senescence and remobilisation

The organisation and integrity of cells during senescence in leaves supporting the grain showed striking changes. Leaves supporting the developing grain at early milk stage, just before the start of grain filling, showed densely packed mesophyll parenchyma cells and vascular bundles (Fig. 8a). As the leaf proceeded through active mobilisation of assimilate to support grain filling, the leaf sections showed a

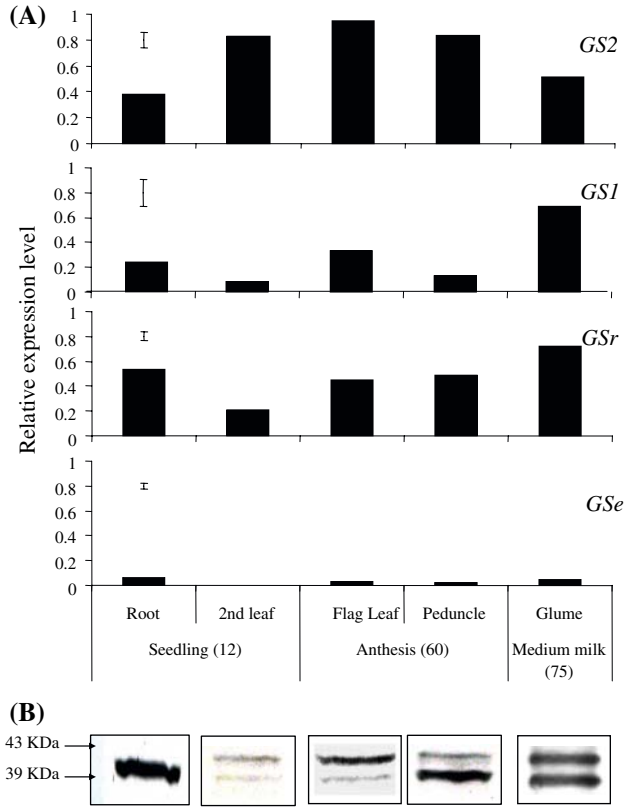

Fig. 6 Tissue distribution of GS mRNA and polypeptides in wheat. (**a**) GS mRNA expression using TaqMan assays with specific probes for each GS gene subfamily. The calibrator was a leaf replicate sample taken at booting stage Zadoks 50. Data are given as the mean of four replicate plants with l.s.d at 5%. Numbers in parenthesis on the *x*-axis correspond to the development stage on the Zadoks scale. (**b**) Western blots of tissue extracts probed for GS polypeptides, tracks were loaded with 15 μg soluble protein. The results are representative of three replicates

large loss in the number of parenchyma cells but with some visible chloroplasts still present in the remaining mesophyll cells (Fig. 8b). At this stage of leaf development, there was no corresponding loss in xylem or phloem cells. As the leaf moved further into senescence, additional losses occurred in parenchyma cell structure and organisation with some changes in the size of vascular cells (data not shown).

GS profile in three genotypes at grain filling

The profile of GS mRNA, protein and activity was studied in doubled haploid members of a mapping population with similar flowering and grain filling time (DH line 90 and 91) and in another cultivar Cadenza. Leaves were sampled from post-anthesis plants at the end of grain filling at Zadoks stage 85. The profile of GS mRNA abundance was different in flag leaves of all three wheat genotypes with *GS2* mRNA levels higher in DH line 91 compared to DH line 90 and Cadenza (Fig. 9a). The amount of *GS1* transcript was similar in both DH lines but tended to be higher, although not statistically significant, in Cadenza whilst *GSr* transcripts levels were similar in Cadenza and DH line 90 but lower in DH line 91. Western blot analysis also showed that the profile of cytosolic and chloroplastic GS polypeptide was different in the leaves of the three wheat genotypes (Fig. 9b). Whilst cytosolic GS was the main isoform present in leaves of both Cadenza and DH line 90, the chloroplastic and cytosolic GS polypeptides were of equal proportion in DH line 91. Measurements of leaf fresh weight (Fig. 9c), soluble protein (Fig. 9e) and GS activity per fresh weight (Fig. 9d) showed no differences between the genotypes, however, there was evidence of a statistically significant higher total GS activity per soluble protein in DH Line 91 (Fig. 9f).

Discussion

Wheat GS gene family

The detailed study of the GS gene family in wheat has lead us to a better understanding of the function of specific GS isoenzymes particularly as it has facilitated the comparison with other plant species. The alignments of wheat GS amino acid sequences highlighted functional residues that are also conserved in GS sequences from other plant species (Fig. 1). The highlighted residues His-249, Asp-56, Glu-297 and Cys-92 have previously been suggested to be involved in, respectively, glutamate binding at the active site (Forde and Cullimore 1989, for review), transferase activity (Clemente and Marquez 1999a), stabilizing a transition state for a reaction subsequent to ammonium binding (Clemente and Marquez 1999b) and thermal stability (Clemente and Marquez 2000). The chloroplastic GS sequence contains two additional Cys residues (positions 303 and 368), which constitute redox response sites that are important for the activity of chloroplastic GS (Choi et al. 1999). Recent structure/function studies in *Arabidopsis thaliana* have shown that the presence of either a Gln residue at position 49 or a Ser residue at position 174 was necessary for high substrate affinity of GS1 isoforms (Ishiyama et al. 2006). If these criteria were applied to wheat sequences, then we would predict that *GS1b*, *GS1c*, *GSr1* and *GSr2* are high affinity isoforms while *GSe1*, *GSe2* and *GS1a* are of low substrate affinity. The X-ray structure of maize GS (Unno et al. 2006) revealed the presence of an Ile at position 161 which conferred heat stability to the GS protein. In wheat, only *GS1* (*a*, *b* and *c*) gene products have the required residue at this position. Our phylogenetic analysis showed that the three wheat cytosolic GS sub-families are in a different clade to the

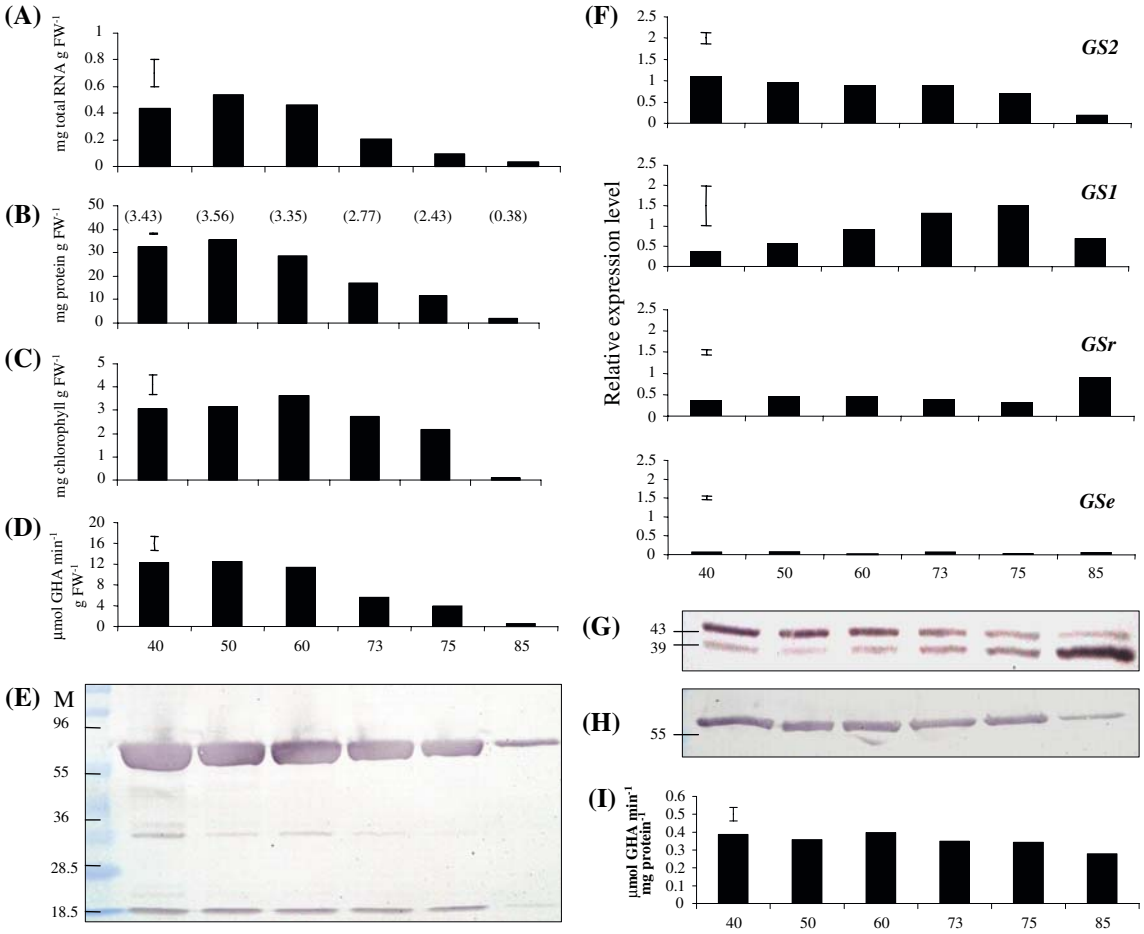

Fig. 7 Flag leaf biochemical parameters throughout plant development. (**a**–**d**) Total RNA, soluble protein, chlorophyll and total GS synthetase activity per leaf fresh weight. Numbers on the *x*-axis refer to stages of plant development according to the Zadoks scale. (**e**) Western blots of leaf extracts probed with Rubisco specific antibodies with tracks loaded on equal leaf fresh weight. (**f**) GS mRNA expression using TaqMan assays with specific probes for each GS gene subfamily. The calibrator was a leaf replicate sample taken at booting stage Zadoks 50. Western blots of leaf extracts probed for GS (**g**) and Rubisco (**h**) polypeptides, tracks were loaded with 5 µg soluble protein. (**i**) Total GS synthetase activity per leaf soluble protein. Westerns are representative of three replicates and all other data are presented as the mean of four replicate plants with l.s.d at 5%. For (**b**), natural log transformation of the data was done and l.s.d should be used to compare mean log values shown in parenthesis above each bar

chloroplastic gene as reported in other species (Doyle 1991; Biesiadka and Legocki 1997). Each of the three clades for cytosolic GS genes contained orthologous genes from the major crop species wheat, maize and rice.

Tissue and cellular specificity of GS in wheat

This is the first report of the localisation of two distinct wheat cytosolic *GS1* and *GSr* transcripts to different cell types in plant tissue at flowering and the results suggest non-overlapping physiological roles. In leaves, *GS1* transcript was localised in the perifascicular sheath specifically in the cytosol of parenchyma and mestome sheath cells surrounding the vascular bundles thus supporting preliminary immunolocalisation studies of GS protein in wheat (Kichey et al. 2005). These cells are part of a complex cellular system channelling and delivering assimilate to the vascular sieve cells (Kuo et al. 1974; Schulz 1998). The fact that *GS1* is detected in the cytosol of these cells confirms that ammonia assimilation to glutamine is occurring here. However, the origin(s) of this ammonia remain unclear and it could originate from amino acid catabolism (Hirel and Lea 2001) and protein hydrolysis (Hortensteiner and Feller 2002) as macromolecules are dismantled during leaf assimilate remobilisation and senescence. Another source could be due to the action of phenylalanine ammonia-lyase (PAL) in the first step of the phenylpropanoid pathway since mestome cells contain both suberin and lignin at this stage of plant development. Recent work in rice demonstrated co-localisation of cytosolic GS and PAL in immature leaves in rice (Sakurai et al. 2001) but further work is necessary to establish

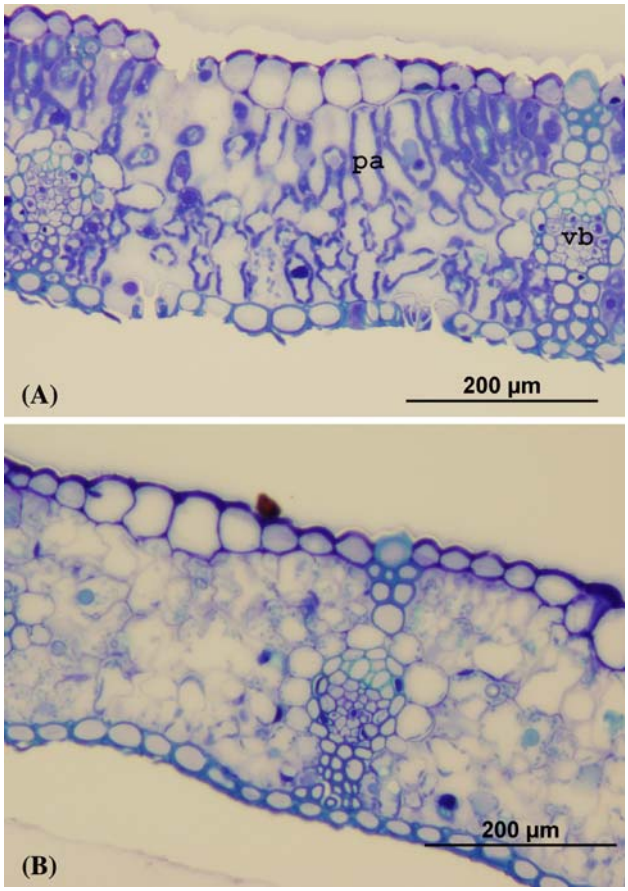

Fig. 8 Morphological and structural observations on leaf sections taken from tillers supporting grain at early milk (Zadoks 73) (**a**) and early dough (Zadoks 83) (**b**). Sections were stained with methylene blue. Pa, parenchyma; vb, vascular bundle

whether the phenylpropanoid pathway is a source of ammonia in mature and remobilising wheat leaves.

Our data, in all tissue, shows distinct localisation of *GSr* mRNA in the companion cells to the phloem sieve cells and in the parenchyma adjacent to xylem cells. These cells contain mitochondria and are thus able to supply the ATP necessary for glutamine production. It has also been shown in rice that NADH-GOGAT is also present in metaphloem and metaxylem cells, thus completing the GS-GOGAT cycle necessary for glutamine production via GSr and subsequently ready for loading directly into the vascular system. The source(s) of ammonia are undetermined but similar immunolocalisations have been recorded for cytosolic GS protein in rice (Sakurai et al. 1996) and we propose, from our phylogenetic and in situ results in wheat, that the immunolable label observed in rice, in these latter studies, in vascular cells is a product of the orthologous *GSr* gene *Os-Gln1;2*.

The four wheat GS sub-families differentially expressed in all tissues show similar profiles to those found in orthologous GS genes in rice and maize (Ishiyama et al.

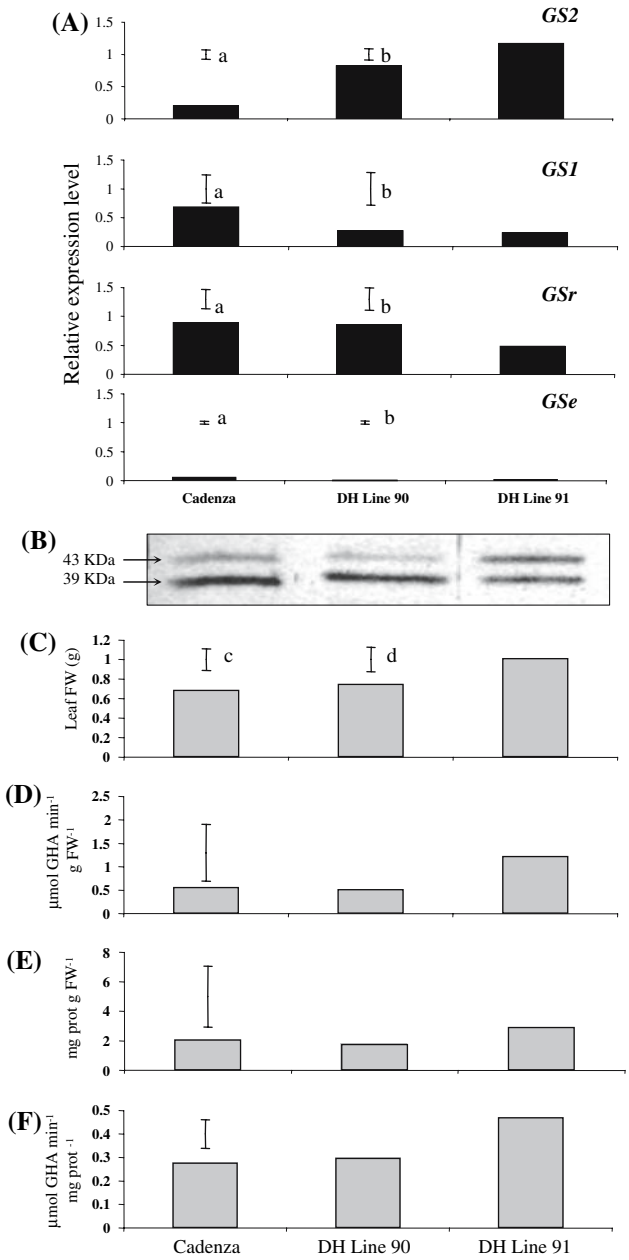

Fig. 9 Flag leaf biochemical parameters and profile of GS genes in leaves of three wheat genotypes at Zadoks 85. (**a**) GS mRNA expression using TaqMan with specific probes for each GS gene subfamily. The calibrator was a leaf replicate sample taken at medium milk stage Zadoks 75. (**b**) Western blot analysis of leaf samples with 5 µg of protein loaded per track; data is representative of three replicate samples. (**c**) Leaf fresh weight. (**d**) Total GS synthetase activity per leaf fresh weight. (**e**) Leaf soluble protein per leaf fresh weight. (**f**) GS synthetase activity per leaf soluble protein. (**b**–**f**) Data are presented as the mean of four replicate plants with l.s.d at 5%. For (**a**), two l.s.d values were generated from the ANOVA, the l.s.d labelled a for data analysis between Cadenza and DH line 90 and DH line 91, l.s.d labelled b for the data analysis between DH line 90 and DH line 91. Similarly for (**c**), the l.s.d labelled c was generated from the data analysis between Cadenza and DH line 91 while l.s.d labelled d was generated from the data analysis between DH line 90 and Cadenza and DH line 91

2004a, b; Sakakibara et al. 1992; Li et al. 1993) however, there are some interesting differences. Maize *Gln1-3* and *Gln1-4*, which corresponds to the wheat *GS1* clade (Fig. 2), have recently been localised to the cytosol of mesophyll and bundle sheath cells respectively (Martin et al. 2006). In our study, *GS1* is found only in the cytosol of parenchyma and mestome cells specific to the perifascicular sheath surrounding the vascular cells with no label observed in the mesophyll cytosol. The significance of this difference in the localisation between maize and wheat may lie in their different C_3 and C_4 cellular and metabolic compartmentation and this distinction requires further exploration.

Overall, our data demonstrate that GS1 and GSr are the major wheat cytosolic isozymes carrying out ammonia assimilation derived from various sources according to the matrix suggested by Miflin and Habash (2002). Further work is required to identify the source(s) of ammonia and the cellular localisation of the third low-expressed GSe isozyme.

Major changes occur in leaf metabolism, cellular organisation and GS after anthesis

Anthesis triggers the start of global changes in wheat leaf metabolism with RNA, soluble protein, chlorophyll, Rubisco subunits and GS2 protein showing co-ordinated and gradual decline (Fig. 7). These changes characterise the onset of senescence and remobilisation of assimilate to the developing grain as defined by Feller and Fisher (1994) and demonstrated in rice (Kamachi et al. 1991). Leaf chloroplastic GS polypeptide varies in line with the amount of Rubisco protein during senescence thus confirming their co-ordinated regulation during grain development and filling in wheat (Fig. 7). *GS2* expression decreased substantially only at medium milk stage (75), a point when the chlorophyll:protein ratio was maximal. Towards the later stages of leaf senescence, GS2 and Rubisco proteins were still detectable on gels and total GS activity could also be measured from such old leaves (Fig. 7i). Studies on natural and induced senescence in leaves have identified a co-ordinated sequence of biochemical and structural events in chloroplast degradation (Feller and Fisher 1994; Hortensteiner and Feller 2002). Wheat leaf sections at the later stages of leaf senescence also showed major losses of mesophyll cells with relatively few chloroplasts still present (Fig. 8b). The xylem and phloem cells remained relatively intact in wheat flag leaves during the grain filling period (Zadoks 73–83) in agreement with other observations (Kichey et al. 2005). This co-ordination ensures that the products of chloroplast and cellular degradation are efficiently transported to the developing sinks. It is important to take into account the ratio of mesophyll to vasculature in the analysis of gene function when pooling cells from tissues undergoing major cellular changes. Similar considerations have recently been highlighted in studies on wheat glumes where a lower mesophyll: vascular cell ratio corresponded to a higher GS1:GS2 ratio with consequences for the profile of nitrogen metabolism when compared to that in flag leaves (Lopes et al. 2006).

We have now shown that the two cytosolic GS genes, *GS1* and *GSr*, contribute to the proportionally higher GS1:GS2 polypeptide ratio during leaf senescence and to the assimilation of ammonia in a complex manner. We have also noted a significant increase in their mRNA at the last phases of grain filling which coincided with the disappearance of mesophyll but not of vascular cells which remained intact (Fig. 8). Combining these results with the specific localisation of these two isozymes in cells surrounding the vascular tissue (GS1) and in cells adjacent to the phloem sieve elements (GSr) supports the idea that they function primarily in assimilating ammonia generated from the various processes involved during the remobilisation of assimilate. This conclusion agrees with that reached in studies on cytosolic GS during leaf senescence in rice (Sakurai et al. 1996). Future work should target the cellular localisation of GS isozymes during the whole process of grain filling and determine the sources and fluxes of ammonia in remobilising and senescing wheat leaves.

Evidence for the regulation of GS

The comparison of transcript and polypeptide abundance in a variety of samples was used to look for evidence of post-transcriptional regulation. In mature leaves, GS transcripts and polypeptides changed in a co-ordinated manner as seen in Fig. 7, suggesting that GS was mainly regulated at the mRNA level. However, some correspondence between the expression of *GS2* mRNA and polypeptide abundance suggests regulation at both the post-transcriptional and post-translational level; such that total GS polypeptide and activity are lower in a seedling leaf compared to a flag leaf for a similar level of mRNA (Fig. 6). The situation for GS1 is more difficult to interpret since several genes may contribute to the protein measured on westerns. Nevertheless, results obtained in this study in different tissues (Fig. 6) and in leaves at various developmental stages (Fig. 7) point to post-transcriptional regulatory steps such as transcript stability. The ORF for *GS1* a, b and *GS2* cDNA was able to complement GLN1 deficient *Saccharomyces cerevisiae* but the full-length cDNA were not, suggesting that the untranslated regions impair their transcript stability (data not shown). Further, our studies showed no significant evidence for diurnal modulation in GS gene expression or enzyme protein or activity in leaves at anthesis (data not

shown) which is similar to results in tobacco (Becker et al. 1992) and rice (T. Yamaya, personal communication) but differs from those in Arabidopsis (Gibon et al. 2004).

Genotypic variation in wheat leaf GS protein

An investigation into the contribution of total flag leaf GS activity to grain yield and nitrogen use was recently undertaken using a bread wheat mapping population derived from the cross Chinese Spring × SQ1 (Habash et al. 2007). This mapping population segregates for many traits including flowering time and the length of the period of grain filling. QTLs for flag leaf total GS activity were positively co-localised with others for grain and stem nitrogen, but smaller correlations were established with loci for grain yield components. Furthermore, some QTLs for GS activity were identified and co-localised to a *GS2* gene mapped on chromosome 2A, and another with the mapped *GSr* gene on 4A. However, the largest QTLs for GS activity were not at the site of mapped GS genes implying other major regulators of this trait; this is not surprising considering the evidence presented here of a highly regulated system for this enzyme family. In this current study, we measured variation in flag leaf GS mRNA, protein and activity in two genotypes which had similar anthesis and grain filling time, to establish whether there is genetic variation in GS proteins in mapping lines which do not segregate for plant developmental rates. The results presented in this study showed that the profile of GS2:GS1 protein in these two select lines was different which resulted in a subsequent variation in the total GS activity per protein but not when calculated on a fresh weight basis. This, in addition to a recent report on two wheat cultivars showing variation for GS activity and yield (Kichey et al. 2006), provides preliminary evidence to support the view that genetic differences exist in leaf GS activity and protein profile in wheat germplasm. This is further backed up by genetic studies in rice (Yamaya et al. 2002; Obara et al. 2004) and maize (Hirel et al. 2001, 2007; Galais and Hirel 2004) demonstrating co-localisations of QTLs for GS protein or activity at the mapped GS genes and with QTLs relating to grain parameters. Integrating the biochemical and genetic approaches to further establish allelic differences in GS isozymes and to uncover new regulatory loci modulating GS activity in diverse genetic material or mapping populations should be targeted in the future. A complementary strategy of manipulating GS by genetic engineering has given mixed phenotypes in several species (Good et al. 2004) and this is probably due to the complexity of regulation of this enzyme family and to the use of inadequately targeted promoters in terms of tissue and developmental specificity. Thus, only by understanding such regulatory elements at the metabolic, cellular and developmental levels and in diverse genetic backgrounds can we then successfully aim genetic engineering strategies to exploit variation in GS as a target for modulating nitrogen use in pre-breeding programmes in wheat.

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